PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
A61K 31/195, 31/395, 31/40

A1

(11) International Publication Number: **WO 87/ 04925**

(43) International Publication Date: 27 August 1987 (27.08.87)

(21) International Application Number: PCT/AU87/00037

(22) International Filing Date: 10 February 1987 (10.02.87)

(31) Priority Application Number: PH 4668

(32) Priority Date: 18 February 1986 (18.02.86)

(33) Priority Country: AU

(71) Applicant *(for all designated States except US):* BIOTA SCIENTIFIC MANAGEMENT PTY. LTD. [AU/AU]; Malleson's, Level 28, North Tower, Rialto, 525 Collins Street, Melbourne, VIC 3000 (AU).

(72) Inventor; and
(75) Inventor/Applicant *(for US only) :* McAUSLAN, Brian, Richard [AU/AU]; 83 Hudson Parade, Clareville, NSW 2107 (AU).

(74) Agent: SANTER, Vivien; Clement Hack & Co., 601 St. Kilda Road, Melbourne, VIC 3004 (AU).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.
With amended claims and statement.

(54) Title: STIMULATION OF ANGIOGENESIS

(57) Abstract

Method for stimulating angiogenesis in a mammal, characterized by the use of a modulator of collagen sysnthesis or of collagen fibril assembly. Preferably the modulator is an inhibitor of the enzyme proline hydroxylase. Preferably the modulator is selected from the group which includes cis-4-hydroxy-L-proline, 3,4-dehydro-L-proline, L-azetidine-2-carboxylic acid, L-proline analogues, and their pharmacologically active analogues and derivatives. The modulator may optionally be used together with a second stimulator of angiogenesis. Compositions containing said modulators are also claimed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**FR**	France	**ML**	Mali
AU	Australia	**GA**	Gabon	**MR**	Mauritania
BB	Barbados	**GB**	United Kingdom	**MW**	Malawi
BE	Belgium	**HU**	Hungary	**NL**	Netherlands
BG	Bulgaria	**IT**	Italy	**NO**	Norway
BJ	Benin	**JP**	Japan	**RO**	Romania
BR	Brazil	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SN**	Senegal
CH	Switzerland	**LI**	Liechtenstein	**SU**	Soviet Union
CM	Cameroon	**LK**	Sri Lanka	**TD**	Chad
DE	Germany, Federal Republic of	**LU**	Luxembourg	**TG**	Togo
DK	Denmark	**MC**	Monaco	**US**	United States of America
FI	Finland	**MG**	Madagascar		

STIMULATION OF ANGIOGENESIS

This invention relates to the control of angiogenesis, and methods and compositions therefor.

According to one aspect of the present invention there is provided a method for stimulating angiogenesis in a mammal, characterized by the use of a modulator of collagen synthesis or of collagen fibril assembly.

Background of the Invention

Knowledge of factors controlling proliferation of the endothelium is essential for understanding the molecular and cellular basis of the normal process of capillary

formation and of pathological processes such as abnormal retinal vasoproliferation leading to blindness, and tumor-induced angiogenesis.

Full identification of the references cited hereinafter will be found at the end of this specification.

By studying the migratory and proliferative responses of cultured endothelial cells it should be possible to identify those substances that might be involved in regulation of neovascularisation. A number of polypeptide growth factors has been shown to enhance vascular endothelial cell proliferation in vitro. These include 3T3-cell derived growth factor (McAuslan et al., 1980), tumor-derived growth factor (Klagsbrun et al., 1982), endothelial cell growth stimulator (ECGF; Maciag et al., 1981), and epidermal growth factor (EGF; Ben-Ezra, 1978; McAuslan et al 1985).

The induction of new blood vessel growth and formation of a vascular network is elicited in animals by extracts of carcinoma cells (Folkman, 1974) or of normal bovine parotid glands (Fleming, 1959). Partially purified fractions of quite low-molecular-weight substances (200-300 Dalton) from Walker carcinoma (McAuslan and Hoffman, 1979; Weiss et al 1979; Fenselau et al., 1981), bovine parotid glands, or bovine liver (McAuslan et al., 1981) have been shown to be angiogenic by ocular implant or chick chorioallantoic membrane assays. It has been shown that low concentrations of copper ions can induce neovascularisation in the anterior eye chamber or corneal pocket and also migration of endothelial cells in culture (McAuslan, 1979; McAuslan and Gole, 1980; McAuslan and Reilly, 1980).

Thus a wide variety of agents has been shown to be capable of inducing angiogenesis in various assay systems. Some of these agents appear to act via a leukocyte-mediated mechanism, since the response is blocked by pretreatment of the test animals with corticosteroids.

It is known that some of the mediators produced in response to an inflammatory stimulus are angiogenic. Because of undesirable side effects of inflammation, an ideal agent

for control of angiogenesis should have a direct action. Although some such agents have been proposed, a mechanism for their action has not been discovered. A further limitation is imposed by the necessity for the agent to penetrate the target organ.

It has been suggested that at least some angiogenic factors induce the synthesis or activity of a protease which attacks collagen, releasing active peptides. In previous studies on collagen synthesis, it was observed that the amino acid cis-4-hydroxy-L-proline (cis-HYPRO) acted as an inhibitor of the enzyme proline hydroxylase, with concomitant inhibition of assembly of collagen fibrils (Tan et al. 1983).

If a "wound" is made by scratching a monolayer of cells grown in tissue culture, the "wound" is soon repaired by migration of cells into the space left by the scratch. Cis-HYPRO will prevent this migration of cells into the wound (Madri and Stenn, 1982).

Surprisingly, and in contrast to the above finding, I have now found that cis-HYPRO will stimulate the migration of capillary endothelial cells <u>in vitro</u>. I have also fund that cis-HYPRO stimulates angiogenesis <u>in vivo</u> in the rabbit corneal pocket assay.

I have found that compounds which stimulate endothelial cell migration <u>in vitro</u> are always angiogenic <u>in vivo.</u> However, because of the role of inflammatory mediators in some angiogenic systems, the converse is not necessarily true. Consequently, as a further confirmation of angiogenic activity, I have used an assay system in which an annular ring of silicone containing a matrix of highly purified atelo-collagen in which is embedded a 1 mm^3 fragment of slow-release copolymer of polyethylene-vinyl acetate impregnated with the agent to be tested is implanted subcutaneously into rabbits. This system is biocompatible and non-inflammatory, and the assay is highly sensitive.

I have also used a direct assay for induction of proliferation of capillary endothelial cells, as described by McAuslan et al (1983), since such proliferation is thought to play a secondary role in angiogenesis.

A completely unexpected novel finding for which I have no explanation is that cis-HYPRO increases the proliferation rate of capillary endothelial cells. Other inhibitors of proline hydroxylase do not appear to do so.

I have further found that other agents which modulate collagen synthesis or which inhibit proline hydroxylase will also stimulate angiogenesis in the above assays. Examples of such compounds include 3, 4-dehydro-L-proline (dHPro), and L-azetidine-2-carboxylic acid (AZET). Other proline analogues may also be useful.

It appears that, in general, agents which modulate collagen synthesis or collagen fibril assembly orwhich inhibit proline hydroxylase will cause the production of defective collagen which is more susceptible to degradation by proteases. Capillary endothelial cells migrate along the gradient of inhibitor concentration, and form capillary tubules in vivo despite the defect in the collagen which is synthesized.

Summary of the Invention

Thus according to one aspect of the present invention there is provided a method of stimulating angiogenesis in a mammal, characterized by the use of a modulator of collagen synthesis or of collagen fibril assembly.

Preferably the modulator is an inhibitor of the activity of the enzyme proline hydroxylase.

More preferably, the inhibitory agent is selected from the group which includes cis-4-hydroxy-L-proline, 3, 4-dehydro-L-proline, L-azetidine-2-carboxylic acid, L-proline analogues, and their pharmacologically active analogues and derivatives.

Combinations of two or more compounds according to the invention may optionally be used.

Combinations of one or more compounds according to the present invention together with one or more compounds according to my copending Australian provisional application PH7521 entitled "Stimulation of Angiogenesis and Control of Endothelialisation" or other stimulators of angiogenesis may also optionally be used.

The compound according to the invention may optionally be administered in a slow-release form or in a biodegradable matrix.

Detailed Description of the Invention

I have used two principal assay systems to test compounds for their ability to stimulate or inhibit angiogenesis. The corneal pocket assay in rabbits as described by Gimbrone et al. (1974) was used according to the modification of McAuslan and Gole (1981). However, in this system it is extremely difficult to distinguish a directly acting angiogenic stimulus from one which is mediated by leukocytes (McAuslan et al., 1983). Since endothelial cell migration is a primary event in neovascularisation, and since there is a correlation between the ability of certain metal ions to induce vascularisation and their ability to cause migration of cultured cells, such migration has been suggested (McAuslan 1979) as the basis for a quantitative assay of angiogenic activity. There is comparatively little information on the correlation between this activity and neovascularising activity, and furthermore, a number of unrelated substances will induce migration of cultured endothelial cells and neovascularisation (McAuslan 1979). Proliferation of endothelial cells is thought to be a response secondary to cell migration during new vessel formation. There are reports of low molecular weight neovasculogenic activities that can stimulate proliferation of cultured endothelial cells. However, the proliferative responses have been marginal and the reports are not in accord as to the

minimal conditions or cell type necessary. Having used one or other of the above assays to purify fractions, they are usually confirmed as angiogenic by an indirect in vivo assay of activity such as the corneal pocket or chorioallantoic membrane assay.

Materials and Methods

Reagents

The proline analogues cis-4-hydroxy-L-proline (cis-HYPRO), 3, 4-dehydro-L-proline (dHPro), cis-4-hydroxy-D-proline (cisdPro) and L-azetidine-2-carboxylic acid (AZET) were obtained from Sigma Chemical Co., St. Louis, U.S.A.. To remove metal ions, they were dissolved in distilled water, applied to a column of Chelex 100 (Biorad), developed in quartz glass double distilled water, then recrystallized. This reduced the copper level to less than 0.06 parts per billion.

Polymer Preparation

Slow-release polymers of polyethylene vinyl acetate (Elvax 60, trade mark of Polysciences Corp.) were prepared by the method of Langer and Folkman (1976). For ocular and subcutaneous assays, sterile fragments of approximately 1 mm^3 were used and for the chorioallantois assay, approximately 2 mm^3.

Rabbit Subcutaneous Assay

An annular ring of silicone containing a matrix of highly purified atelo-collagen Type I (50 mg/ml in buffered saline) in which is embedded a 1 mm^3 fragment of slow-release copolymer of polyethylene vinyl acetate impregnated with the agent to be tested is implanted subcutaneously into each rabbit between the dorsal dermal layer and the muscle fascia.

Each polymer fragment is impregnated with a saturating amount (approximately 0.05 to 0.5 mg) of the solid agent to be tested, so that the agent diffuses out of the polymer and sets up a concentration gradient which changes with time.

Implantation was via a trocar, and was performed so as to avoid trauma to the vascular system. Controls showed no overt inflammatory response and no sign of inducing angiogenesis in surrounding tissue. After 10 days implants were examined in situ, photographed then excised and examined histologically. For each test substance there were at least 2 implants in each of 6 N.Z. white rabbits, one on the left and one on the right dorsal side.

Corneal Pocket Assay

The corneal pocket assay of Gimbrone et al (1974) as modified by Gole and McAuslan (1981) was used on New Zealand white rabbits of 2 - 3 kg body weight. Opposite eyes of each animal were used as control and test, respectively. The results were documented photographically and histologically 10 days postoperation.

Chick Chorioallantoic Membrane Assay

The assay was conducted essentially as described in detail in the review of Gullino (1981) and results recorded at Day 5 to 6. However, I usually found it unnecessary to anchor the polymer fragments (up to 2 mm^3) and I introduced the polymer onto the membrane 1 hour after partially detaching the membrane. In both the corneal pocket assay and this assay it is impractical to determine the effective gradient of the diffusing agents. For this reason only the initial concentrations in the implants are cited.

Cell Lines

Clonal lines of bovine aortal endothelial cells, whose growth and maintenance was as described by McAuslan et al (1982) were used. Similar results were obtained with either type of cell line.

A line of bovine retinal capillary endothelial cells free from mural cells was established essentially by the procedures of Buzney and Massicotte (1979).

Bovine corneal endothelial cell cultures were prepared and maintained as described by McAuslan et al (1979).

Cultures of bovine retinal microvascular pericytes (mural cells) were established from bovine retinal capillaries by the method of Gitlin and D'Amore and maintained in Ham's F12 medium.

All cell lines for experimentation on migration were used between their 8th and 12th passage. Each cell line was tested in its preferred growth medium but with the serum concentration reduced to 2%.

Cell Migration Assays

The procedure for studying induced endothelial cell migration as well as the quantitation of average track lengths has been presented in detail by McAuslan and Reilly (1980).

Briefly, potential migration inducers were added to the medium covering cells, and migration determined by phagokinesis on colloidal gold - bovine serum albumin deposits 16 or 48 hours later. Track areas were estimated using a Bioquant image analysis system (Bioquant is a trade mark of Wild Leitz (Australia) Pty. Ltd.). Results are presented as the means of 25 tracks on each of 3 plates.

Cell Proliferation Studies

Confluence cultures of either aortal or retinal capillary endothelial cells were harvested using 0.25% trypsin in 0.2% Versene (trade mark) and resuspended in growth medium (medium 199 + 5% fetal calf serum + folate) (McAuslan et al 1979); cells were plated onto 60 mm Falcon plastic dishes and allowed to attach for 3 hours before changing the medium. Each test sample was added to replicate dishes. A further addition was made at Day 2 and counts of treated and control cells were determined at Day 4.

Polypeptide Synthesis and Secretion

High density cultures of endothelial cells ($4x10^5$ per cm^2) were treated with cis-HYPRO at 0, 10^{-5} or 5×10^{-4} M for 4 hours in normal growth medium. .The medium was removed and the cell layers were washed and covered with medium containing the appropriate concentration of cis-HYPRO and free of serum. This was supplemented with sodium ascorbate (50 µg/ml) and β-amino propionitrile (80 µg/ml). Then cells were

labelled for [redacted] hours with 20 mCi of [^{14}C[redacted])]-proline (specific activity) per ml of proline free medium 199 (Commonwealth Serum Labs). Polypeptides secreted into the medium were harvested, separated by sodium dodecylsulphate polyacrylamide gel electrophoresis and detected by autoradiography.

The invention will now be illustrated by reference to the following non-limiting examples, together with the accompanying drawings, in which:

Figure 1 represents the effects of proline analogues on the growth of bovine aortal endothelial cells;

Figure 2 represents the effects of proline analogues on the growth of bovine retinal capillary endothelial cells;

Figure 3 represents the effects of proline analogues on the growth of bovine retinal microvascular pericytes;

Example 1

Stimulation of Cell Proliferation

Cultures of bovine retinal endothelial cells, bovine aortic endothelial cells, and bovine retinal pericytes (mural cells) were prepared as described above, and tested separately for their response to cis-HYPRO, cis-4-hydroxy-D-proline, and L-azetidine-2-carboxylic acid. The results were normalised for each experiment as follows:

1. At each reading, the control was assigned a value of 100%. The test value was expressed as a % increase or decrease, e.g. if control = 3×10^{-4} cells/mm^2 = 100%

test = 6×10^{-4} cells/mm^2

test = 200%.

2. Arbitrary control values were calculated using control data for each experiment and the known cell doubling time for each cell line.

e.g. Cell doubling time = 24 hr.

Initial seeding rate = 3×10^{-4} cells/mm^2

At day 2, cell number = 1.2×10^{-4}

= 100%

These values were used to plot the control line for each graph, converting results back to cell counts.

3. Values for test results were converted back to cell counts, using the control value calculated in (2) above as 100%.

e.g. % at day 2 = 200%

Calculated control at day 2 = 100%

$= 1.2 \times 10^{-4}$ cells/mm^2

Cell count $- 2.4 \times 10^{-4}$ cells/mm^2

The results are shown in Figures 1 to 3, in which the test results calculated in (3) and the control results calculated in (2) above are plotted against time in culture. In the figures BREC represents bovine retinal endothelial cells, BAE represents bovine aortic endothelial cells, and BRPC represents bovine retinal microvascular pericytes.

There is no essential link between migration and proliferation. Because of the relevance of cell migration to blood vessel proliferation, it was of interest to know if Cis-HYPRO, dHPro or AZET affected cell proliferation at the concentration optimal for migration induction. The results in Figures 1 to 3 show that at the concentration tested, these agents were not toxic to cells and caused no significant change in the rate of proliferation over at least seven days for BAE or BREC cells.

Example 2

Induction of Cell Migration

A range of concentrations of cis-4-hydroxy-L-proline, 3, 4-dehydro-L-proline (dHPro) and cis-4-hydroxy-D-proline (cisDPro) and L-azetidine-2-carboxyic acid (AZET) was tested for ability to stimulate the migration rate of different cultured cells. AZET and dHPRO are known inhibitors of proline hydroxylase (Kerwar et al, 1975) and all three agents also interfere with synthesis and secretion of collagens (Cardinale and Udenfriend, 1974). An exception is CisDPro which is not incorporated into collagen.

endothelial cells and retinal pericytes are shown in Table 1, in which each figure represent the mean value for 200 individual cells analysed 48 hours after the addition of the test compound. The medium was Ham's F12 medium containing 5% serum (v/v). Each agent was used at a concentration of 10^{-5}M.

Table 1

Effect of agents on cell migration

Agent	Retinal Endothelial Cells		Retinal Pericytes	
	Track Area ($\mu m^2 x 10^{-3}$)	Track Length (μm)	Track Area ($\mu m^2 x 10^{-3}$)	Track Length (μm)
Control	13	152	7	106
cis-HYPRO	46.4	258	21.4	180
cis-D-hydroxyproline	13	152	-	-
azetidine	15.5	172	6.8	106
dehydroproline	17.7	94	17.2	170

Cis-HYPRO strongly stimulated migration of both cell types, while cis-D-hydroxyproline had no effect, and azetidine had a marginal effect on retinal endothelial cells; dehydroproline had a small effect on retinal endothelial cells, and a stronger effect on retinal pericytes.

The results of a second experiment, presented in Table 2, show that cis-HYPRO caused a significant increase in the rate of migration of retinal endothelial cells (BREC), aortal endothelial cells (BAE), corneal endothelial cells (BCE), and retinal pericytes (BRPC). The optimal concentration of inducers for maximal migration rate was of the order of 10^{-5}M at which up to 2-3 fold increases in migration rates were achieved. The nature of the assay preclude a more accurate measurement of the optimal concentration. Similar results were obtained with AZET and dHPro. CisDPro was not active. At concentrations of inducers above 10^{-5}M there was a relative decrease in the rate of migration attained.

Table 2

Migration rate in response to proline analogues

Cell Type	Concentration of inhibitor	Track Area ($um^2 x10^{-3}$) CisHYPRO	CisDPro	AZET	dHPro
BREC	$5x10^{-4}$	20.0	25.2	18.2	18.0
	$1x10^{-4}$	29.2	23.3	30.1	30.5
	$1x10^{-5}$	46.4	24.2	37.9	48.1
	$1x10^{-6}$	28.4	25.0	30.0	32.3
	$1x10^{-7}$	24.2	24.7	25.0	25.0
	0	25.2	25.2	25.2	25.2
BCE	$5x10^{-4}$	30.0	29.8	29.5	30.5
	$1x10^{-4}$	90.1	32.3	50.1	50.1
	$1x10^{-5}$	115.8	36.8	74.6	87.7
	$1x10^{-6}$	36.4	38.6	43.4	45.1
	0	40.0	40.0	40.0	40.0
BRPC	10^{-5}	21.3	6.7	13.5	12.1
	0	6.7	6.7	6.7	6.7
BAE	10^{-5}	17.3	8.5	21.0	14.6
	0	8.5	8.5	8.5	8.5

Example 3

In Vivo Angiogenic Assay

Cis-HYPRO and azetidine were assayed in rabbits using the corneal pocket assay and the subcutaneous assay as described above. The results are presented in Table 2. The figures represent the ratio of animals giving a positive angiogenic response to the total number of animals tested.

Table 3

In Vivo angiogenic response

	Corneal Pocket Assay	Subcutaneous Assay
Control	0/6	0/6
cis-HYPRO	6/6	6/6
azetidine	0/3	3/6

Cis-HYPRO caused stimulation of angiogenesis in both systems, whereas azetidine caused stimulation only in the subcutaneous assay. However, responses in the corneal pocket assay were weak, and the subcutaneous assay was found to be reproducible and effective for a number of chemically different angiogenic agents. When either HYPRO or AZET were tested in a further experiment using this assay, both gave positive angiogenic responses in each of 12 tests in 6 different test animals. dHPro was not found to be active. These results are summarized in Table 4.

Table 4

Subcutaneous implant assay for angiogenesis

Inhibitor	No. of implants/Intensity of vascularisation[a]				
	++++	+++	++	+	-
Control (no inhibitor)	0	0	1	1	10
Cis-HYPRO	4	5	1	2	0
AZET	4	3	2	2	1
dHPro	0	0	1	3	8

[a] Intensity of Vascularisation

++++ Large numbers of distinct capillary vessels invading the gel; numerous blood vessels growing towards the tube. Markedly angiogenic.

+++ Fine blood vessels invading the gel. Less intense than above; fine blood vessels around the silicon tube. Strongly angiogenic.

++ Few fine capillaries around gel periphery, causing a slight pink appearance; fine blood vessels around silicon tube. Weakly angiogenic.

+ Collagen gel unchanged; fine blood vessels growing towards silicon tube; incipient angiogenesis.

- Collagen gel unchanged; no blood vessels around silicon tube. Non-angiogenic.

Example 4

Cis-HYPRO as an Enhancer of Epidermal Growth Factor

The angiogenic activity of epidermal growth factor, (EGF; Ben-Ezra, 1978) can be explained by its ability to induce capillary endothelial cell migration and proliferation

(McAuslan et al, 1985). However EGF is found to be a highly potent inducer and is less active angiogenically than other growth factors such as transforming growth factor TGF- (Schreiber et al, 1986). Therefore we tested the possibility that the responses produced by Cis-HYPRO and EGF might be additive. The induction of bovine corneal endothelial cell migration by EGF, Cis-HYPRO or EGF plus Cis-HYPRO was compared. As shown in Table 5, the migration-inducing ability of EGF and Cis-HYPRO was at least additive over the limited range tested.

Table 5

Effect of cis-HYPRO on stimulation of angiogenesis by Epidermal Growth Factor

	Bovine corneal endothelial cell migration $10^{-3}\mu m^2$/24 hours
Control	35.8
EGF (100µg/ml)	116.0
cis-HYPRO (10^{-5}M)	56.4
EGF (100 µg/ml) + cis-HYPRO (10^{-5}M)	266.7

Thus cis-HYPRO or other proline analogues could be used to enhance the angiogenic effect of EGF, or of the active subunit of EGF which is responsible for angiogenesis.

Example 5

Effect of Cis-HYPRO on Synthesis of Collagenous Polypeptides

Since there was a marked fall in the migration rate achieved when the concentration of inhibitors was increased from 10^{-5}M to $5x10^{-4}$M, I compared the levels of [^{14}C]-labelled polypeptides, precipitable by trichloracetic acid (10% w/v), from the medium used to maintain cells. Cells treated with $5x10^{-4}$M Cis-HYPRO secreted 60% (59,600 cpm/ml) of the level

secreted by control cultures (98,700 cpm/ml) while cells treated with the lower level of Cis-HYPRO (10^{-5}M) secreted 80% (80,800 cpm/ml) as compared to control cultures.

The above experiments show that random cell migration in vitro, as measured by increase in the area of phagokinetic tracks produced in a colloidal gold deposit, was increased significantly by Cis-HYPRO, dHPro or AZET. Both vascular and avascular endothelial cells responded, as did retinal pericytes. Thus this is a regulatory mechanism that may be common to many anchorage-dependent cell types.

These experiments were initiated on the premise that the agents tested are known inhibitors of proline hydroxylase or of collagen synthesis and secretion and that interference with collagen synthesis might enhance migration. The migratory response (Tables 1 and 2) occurs over only a limited range of concentrations of inducers. Madri and Stenn (1982) showed that the endothelial cell migration studied by a cell monolayer wounding technique, and synthesis of collagen Types IV and V were inhibited by Cis-HYPRO, from which they concluded that continual collagen production is essential for cell migration. In the present experiments the optimal concentration for migration induction (10^{-5}M) was about 40 fold less than used by Madri's group to inhibit migration and block collagen IV formation.

This raises the question of the mode of action of proline analogues as migration inducers. Their action on collagen synthesis at the cellular level is complex. The key question is why Cis-HYPRO (or other proline analogues) should induce cell migration at such low concentrations (10^{-5}M) whereas higher concentrations ($5x10^{-4}$M) were non-inducing or even inhibitory. Without wishing to be bound by any proposed mechanism for the observed beneficial effects, I conclude that whereas continuous collagen synthesis might be needed for cell migration (Madri and Stenn, 1982) at low analogue levels, either a small change in collagen structure occurred which was sufficient to impair matrix assembly and destabilise

anchorage, or at some other specific critical controlling step(s) for cell migration is highly sensitive to low concentrations of the analogue inhibitors.

Since chemically different proline analogues were effective and since there is some steric specificity as evidenced by the lack of activity of CisDPro, it is unlikely that migration induction was due to some migration inducing contaminant such as copper or selenium. Whether Cis-HYPRO and other analogues work as positive effectors (e.g. causing formation of a migration peptide or enhancing the activity of a specific protease) or as negative effectors (e.g. by impairing formation of a critical anchorage component or by blocking synthesis of a migration inhibitor) remains to be established.

While the detailed mechanism of action and cellular responses remains to be resolved the conclusion from these results is that for the microvascular system perturbation of collagen related systems by low concentrations of proline analogues is sufficient to provoke cell migration and angiogenesis.

Applications of the Invention

The present invention is capable of application in a wide variety of clinical fields.

Stimulation of angiogenesis can be used to enhance the healing of burns and wounds, especially those involving large tissue defects, acceptance of skin or organ grafts, and can also be used in reconstructive and cosmetic surgery, including the use of subdermal implants, and in prosthetic surgery, particularly that involving vascular prostheses. Such stimulation may be used in any situation wherein endothelial cell migration and regeneration of endothelium are advantageous, or where an increase in blood flow is desirable, e.g., stroke, heart disease, or foetal blood insufficiency.

This application of cis-HYPRO excludes its use as an agent which might improve the performance of implantable prosthetic devices such as pacemaker electrodes by virtue of properties other than stimulation of angiogenesis.

It will be clearly understood that the invention in its general aspects is not limited to the specific details referred to hereinabove.

References cited herein are listed on the following pages.

REFERENCES

Ben-Ezra, D. (1978) Amer. J. Ophthalmol. 86 455.

Buzney, S.M. and S.J. Massicotte (1970) Invest. Ophthalmol. Visual Sci. 18 1191-1195

Cardinale, G.J. and S. Udenfriend (1974) Adv. Enlymol. 41 245.

Fenselau, A., S. Watt, and R.J. Mello (1981) J. Biol. Chem. 256 9605-9611

Fleming, H.S. (1959) J. Dent. Res. 38 374-385

Folkman, J. (1974) Adv. Cancer Res. 19 331-358

Gimbrone, M.A., R.S. Cotran, S.B. Leapman and J. Folkman (1974) J. Nat. Cancer Inst. 52 413-427

Gitlin. J.D. and P.A. D'Amore (1983) Microvasc. Res. 26 74.

Gullino, P.M. (1981) in Tissue Growth Factors ed. R. Baserga; Springer-Verlag, N.Y. p. 427-449

Kerwar, S.S., R.J. Marcel, and R.A. Salvador (1975) Biochem. Biophys. Res. Comm. 66 1275.

Klagsbrun M., R. Sullivan, P. D'Amore, K. Butterfield and J. Folkman (1982) J. Cell Biol. (Abstract 9091) 95 201

Langer, R and J. Folkman (1976) Nature 263 797-799.

McAuslan, B.R. (1979) EMBO Workshop on Specific Growth Factors, Rome, Oct. 9-11.

McAuslan, B.R., V. Bender, W. Reilly, and B.A. Moss (1985) Cell Biol. Int. Reports 9 175.

McAuslan, B.R. and G.A. Gole (1980) Trans. Ophthalmol. Soc. U.K. 100 (3) 354-358

McAuslan, B.R. G.N. Hannan, and W. Reilly (1980) Exptl Cell Res. 128 95-101

McAuslan, B.R. and H. Hoffmann (1979) Exptl Cell Res. 199 181-190

McAuslan, B.R. and W. Reilly (1980) Exptl Cell Res. 130 147-157

McAuslan, B.R., W. Reilly and G.N. Hannan, J. Cell Physiol (1979) 100 87.

McAuslan, B.R., W. Reilly and G.N. Hannan (1981) in Progress in Microvascular Research (ed. D. Garlick) p.470-501

McAuslan, B.R. W. Reilly, G.N. Hannan, and G.A. Gole (1983) Microvascular Res. 26 323-328

Maciag, TO., A. Hoover, M.B. Stemmerman, and R. Weinstein (1982) J. Cell Biol. 91 420-426

Madri, J.A. and K.S. Stenn (1982) Am. J. Pathol. 106 180-186

Schreiber, A.B., M.E. Winkler, and R. Derinck (1986) Science 232 1250.

Tan, E.M.L., L. Ryhanen, and J. Uitto .(1982) J. Invest. Dermatol. 80 261-267

Weiss, J.B., [redacted]. Brown, S. Kumar, and P[redacted]hillips (1979) Brit. J. Cancer 40 493-496.

CLAIMS:

1. A method of stimulating angiogenesis in a mammal, characterized by the use of a modulator of collagen synthesis or of collagen fibril assembly.

2. A method according to Claim 1, wherein said modulator is an inhibitor of the activity of the enzyme proline hydroxylase.

3. A method according to Claim 1, wherein said modulator is selected from the group which includes cis-4-hydroxy-L-proline, 3,4-dehydro-L-proline, L-azetidine-2-carboxylic acid, L-proline analogues, and their pharmacologically active analogues and derivatives.

4. A method according to any one of Claims 1 to 3, wherein two or more of said modulators are used.

5. A method according to any preceding claim, wherein said modulator is used together with a second stimulator of angiogenesis.

6. A method according to Claim 5, wherein said second stimulator is an anti-inflammatory agent.

7. A method according to Claim 6, wherein said anti-inflammatory agent is selected from the group which includes salicylic acid, anthranilic acid, phenyl acetic acid, and thiazole acetic acid, and their pharmacologically active analogues and derivatives.

8. A method according to Claim 5, wherein said second stimulator of angiogenesis is epidermal growth factor or a pharmacologically active analogue or fragment thereof.

9. A method according to any preceding claim wherein said modulator is administered so as to achieve a diffusion gradient of concentration to which endothelial cells respond.

10. A method according to any preceding claim, wherein said modulator is administered in a slow-release form or in a biodegradable matrix.

11. A method according to any preceding claim wherein the concentration of said modulator is 10^{-7} to 10^{-4} mole/l.

12. A composition for stimulation angiogenesis comprising as effective component a modulator of collagen synthesis or of collagen fibril assembly, together with a pharmaceutically acceptable carrier or diluent.

13. A composition according to Claim 12, wherein the modulator is selected from the group which includes cis-4-hydroxy-L-proline, 3,4-dehydro-L-proline, L-azetidine-2-carboxylic acid, and their pharmacologically active analogues and derivatives.

14. A composition according to Claim 12 or Claim 13, comprising two or more of said modulators.

15. A composition according to any one of Claims 12 to 14, which additionally comprises a second stimulator of angiogenesis.

16. A composition according to Claim 15, wherein said second stimulator is an anti-inflammatory agent.

17. A composition according to Claim 16, wherein the anti-inflammatory agent is selected from the group which includes salicylic acid, anthranilic acid, phenyl acetic acid, and thiazole acetic acid, and their pharmacologically active analogues and derivatives.

18. A composition according to Claim 15, wherein the second stimulator of angiogenesis is epidermal growth factor or a pharmacologically analogue or active fragment thereof.

19. A composition according to any one of Claims 12 to 18 wherein the modulator is present at a concentration of 10^{-7} to 10^{-4} mole/l.

20. A composition according to any one of Claims 12 to 19 wherein the modulator is compounded into a slow release form or into a biodegradable matrix.

21. A method of stimulating angiogenesis in a mammal, substantially as hereinbefore described with references to the examples.

22. A composition for stimulating angiogenesis in a mammal, substantially as hereinbefore described with reference to the examples.

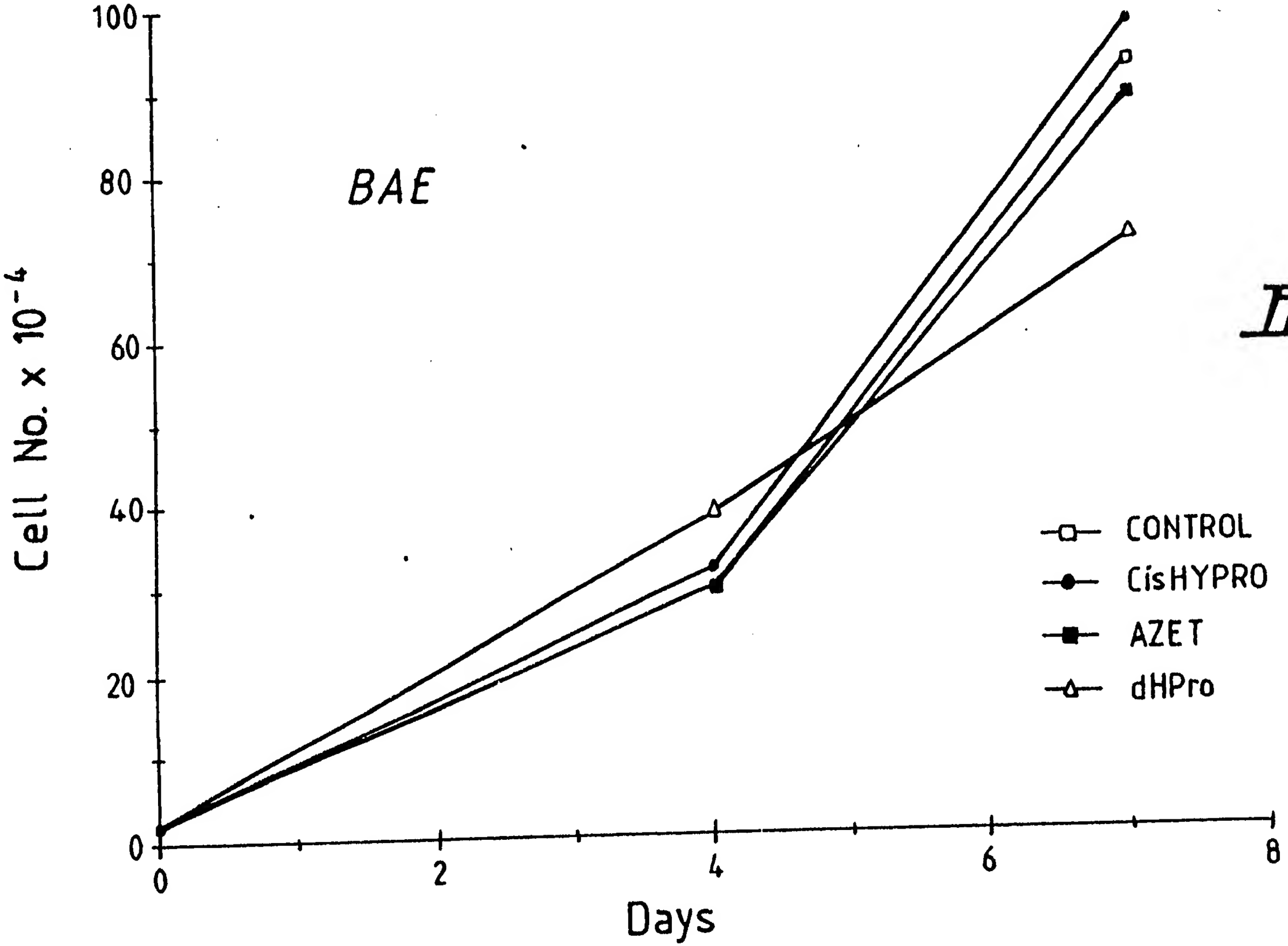


FIG. 1.

1/3

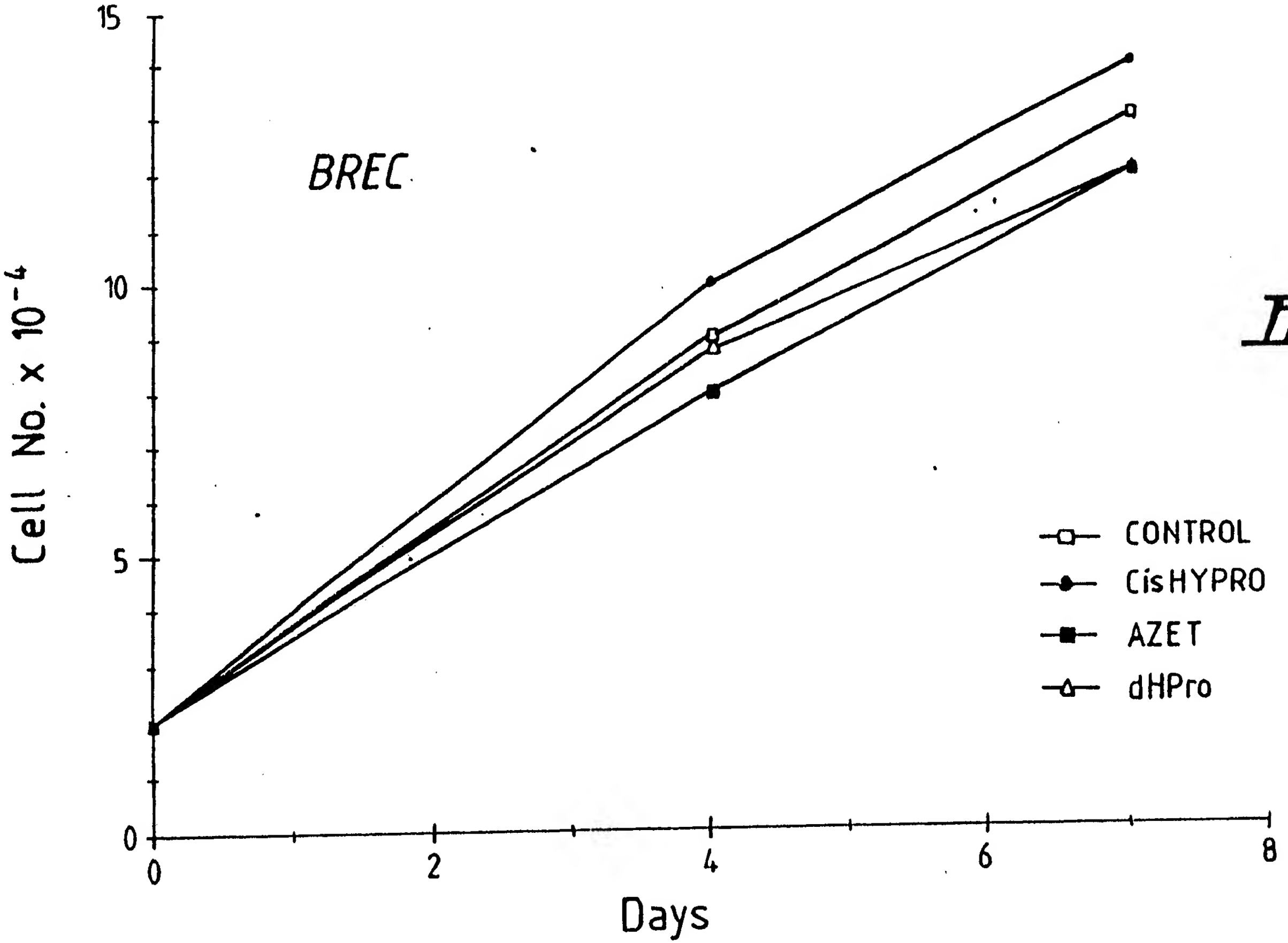
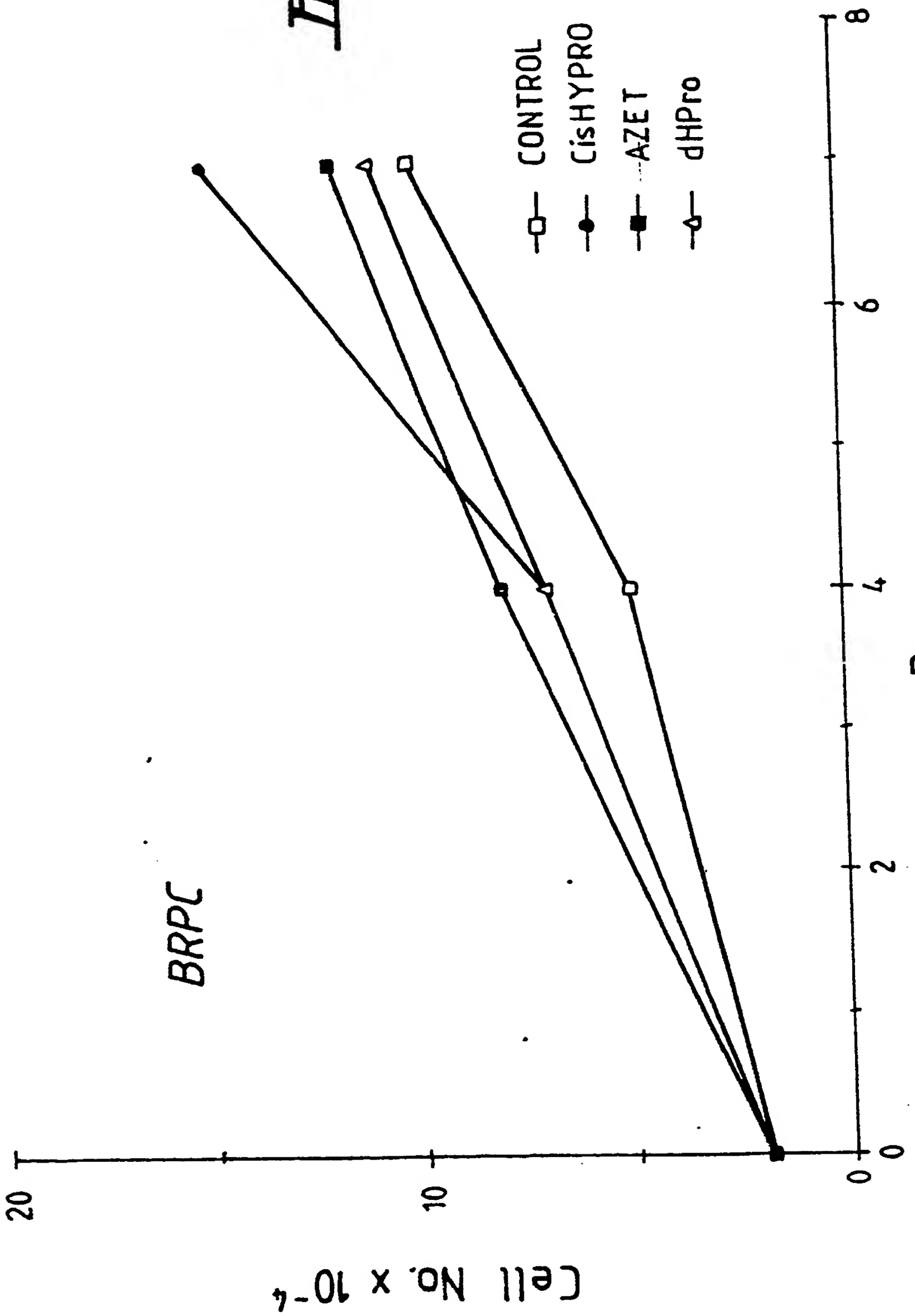


FIG. 2.

3/3

FIG. 3.



INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00037

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] A61K 31/195, 31/395, 31/40

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	A61K 31/195, 31/395, 31/40

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU : IPC as above; Australian Classification 87.16

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,A	GB,A, 1399887 (PROCKOP, Darwin Johnson) 2 July 1975 (02.07.75)	1-22
X,A	AU,A, 43369/72 (PROCKOP, Darwin Johnson) 20 December 1973 (20.12.73)	1-22
A	WO,A, 86/07053 (HOERRMANN, Wilhelm) 4 December 1986 (04.12.86)	1-22
A	GB,A, 2171302 (HOERRMANN, Wilhelm) 28 August 1986 (28.08.86)	1-22
A	GB,A, 1246141 (COIRRE, Paul and COIRRE, Bertrand) 15 September 1971 (15.09.71)	1-22

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 May 1987 (25.05.87)	(09.06.87) 9 JUNE 1987
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J.P. PULVIRENTI

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00037

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members			
GB 1399887	BE 784650	DE 2228187	FR 2140598	
GB 1246141	CH 496690	CH 496692	DE 1795327	
	ES 358327	NL 6812996	US 3891765	
	US 3932638	US 3997559	BE 773986	
GB 2171302	DE 3538619	JP 61155324		
WO 8607053	DE 3518078			

END OF ANNEX